Biofilm Formation by *Enterococcus* species of Bovine Mammary Gland and Environmental Origins

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ABSTRACT

In vitro biofilm formation was compared among *Enterococcus* species of bovine origin. One hundred-seventeen enterococcal isolates were tested. Isolates originated from aseptically collected bovine quarter milk samples and bedding samples from a single commercial dairy. Isolates from bovine quarter milk samples originated from mammary glands with clinical mastitis, cows with high somatic cell counts, and postpartum milk samples. Bacterial species tested were *Enterococcus faecium* (n=80), *Enterococcus casseliflavus* (n=28), and *Enterococcus faecalis* (n=9). The bacterial species significantly contributed to the ability of organisms to produce biofilm. *Enterococcus faecalis* biofilm assay values were greater than for either *E. faecium, E. casseliflavus,* or negative controls. Biofilm results did not differ among *E. faecium, E. casseliflavus,* or negative controls. Origin of isolates did not affect biofilm formation. Assay values were comparable among isolates of bovine mammary gland origin and those of isolates from the environment. The ability to form biofilm in vitro does not appear to a pathogenicity factor for *Enterococcus* species associated with bovine intramammary infections.

INTRODUCTION

Enterococcus species often produce biofilms or large communities of bacteria living on a solid surface within a self-produced matrix (5). The production of biofilm in vitro by *Enterococcus faecalis* isolates from human clinical settings was correlated with pathogenicity and virulence of strains (4). The percentages of *Enterococcus faecalis* isolated from either infections or indwelling medical devices producing biofilm ranged from 94 to 100% (1).

Enterococci are capable of infecting humans, companion animals, and livestock. Enterococci are

normal gastrointestinal flora of dairy cows, but are also opportunistic pathogens that can cause bovine mastitis. Enterococci can infect humans and domestic animals because of their many virulence factors associated with biofilm formation including gelatinase, aggregation substance, capsule formation and enterococcal surface protein. Many strains are resistant to one or more antibiotics, including vancomycin (2). Biofilms are thought to contribute to this resistance. The three most commonly isolated species of enterococci from human and bovine clinical samples are *E. faecalis*, *E. faecium*, and *E. casseliflavus*.

Unlike strains from clinical human settings, enterococcal strains isolated from bovine mammary glands, bedding, manure and feed have not been tested for biofilm formation. In vitro growth responses of enterococci in mammary secretions were different between isolates from the bovine mammary glands and those from the environment (3). The relationships between biofilm formation by enterococci and indirect and direct measures of pathogenicity within bovine mammary glands are undefined.

OBJECTIVES

1) compare in vitro biofilm formation between isolates of bovine and environmental origins.
2) compare in vitro biofilm formation among *Enterococcus* species

MATERIALS AND METHODS

Isolates

A total of 117 enterococcal isolates of bovine origin from a single commercial herd of approximately 1200 lactating cows managed in shaded dry lot corrals were tested for in vitro

biofilm formation. The number of isolates by bacterial species and origin are in Table 1. Isolates of bovine origin from the commercial herd were from quarter milk samples collected using aseptic technique from high somatic cell count milk (**SCC)**, mammary quarters with clinical mastitis prior to antibiotic therapy (**CM**), and cows within 3 days after parturition (**Fresh)**. Isolates tested were also from dried manure bedding samples (**Bedding**) collected monthly from the commercial herd.

TABLE 1. *Enterococcus* species isolates tested for in vitro biofilm formation.

Origin of isolates*	*Enterococcus faecium*	*Enterococcus casseliflavus*	*Enterococcus faecalis*	TOTAL
Bedding	17	9	5	31
SCC	31	12	0	43
Clinical Mastitis	21	1	1	23
Fresh	11	6	3	20
TOTAL	80	28	9	117

*Bacterial isolates were from quarter-milk samples of cows in a commercial herd collected using aseptic technique. Milk samples were from cows with high somatic cell count milk (**SCC)**, mammary quarters with clinical mastitis prior to antibiotic therapy (**Clinical Mastitis**), and cows within 3 days after parturition (**Fresh)**. Isolates tested were also from dried manure bedding samples (**Bedding**) collected monthly from the commercial herd.

Biofilm Assay

Bacterial isolates were stored at -80 C in trypticase soy broth containing 10% glycerin prior to

testing. Each isolate was subcultured on trypticase soy agar containing 5% bovine blood and 0.01 % esculin for 24 hours at 37 C. One isolated colony from of each isolate was inoculated into 5 ml of trypticase soy broth (**TSB**) and incubated 16 hours at 37 C and 50 rpm. The broth culture was centrifuged (6000 X *g* for 10 minutes), resuspended to original volume in TSB, and diluted 1:40 in the assay test medium of TSB plus 1.0% dextrose. The assays were performed in 96-well flat bottom polystyrene microtiter plates with 240 µl capacity in each well. Each of eight vertical wells was inoculated with a total of 200 µl of an isolate to allow testing of 11 isolates per plate. Eight vertical wells of each plate contained sterile test medium as negative controls. Microtiter plates were incubated in a humid chamber at 37 C for 24 hours, medium aspirated, and wells washed 3X in phosphate buffered saline. Plates were inverted and allowed to air dry for 1 hour. Aqueous crystal violet was added to each well (200 µl) for 15 minutes at room temperature. The crystal violet was aspirated and wells washed 3X with sterile phosphate buffered saline. Crystal violet was extracted from adhering bacterial cells by addition of 200 µl of 80:20 ethyl alcohol:acetone. The optical density of extracted crystal violet was measured at 630 nm.

Statistics

The main effects of bacterial species, origin of isolates and the interaction bacterial species X origin of isolates were tested by analysis of variance. Multiple comparisons among means were by Tukey's multiple comparison test.

RESULTS AND DISCUSSION

Production of biofilm differed among *Enterococcus* species, but was similar among bacteria from different sources of origin. *Enterococcus faecalis* had greater biolfilm mean assay values than did either *E. faecium* or *E. casseliflavus* **(Figure 1)**. These results agreed with previous

studies that indicated the incidence was higher for biofilm formation among *E. faecalis* strains compared with other enterococcal species. *Enterococcus faecalis* is the species most commonly infecting indwelling medical devices (4). The formation of biofilm by clinical isolates of *E. faecalis* was described as a virulence factor necessary for colonization of prosthetic devices in humans (1). In contrast, *E. faecium* was the most commonly isolated bacterial species from mammary glands and bedding in the current study. Biofilm formation did not appear to be a prerequisite for colonization of the bovine mammary gland. Bacteria from the bedding environment had similar biofilm assay values as did those isolated from mammary glands **(Figure 2)**. Biofilm assay values did not differ between the negative controls and those for bacteria from the mammary glands when all bacteria or only *E. faecium* (**Figure 3)** were compared. *Enterococcus* species are described as "environmental opportunist" in the bovine mammary gland (3). Characteristic of environmental opportunist pathogens is the frequency of many virulence factors in clinical isolates is similar to the frequency in isolates in the environment.

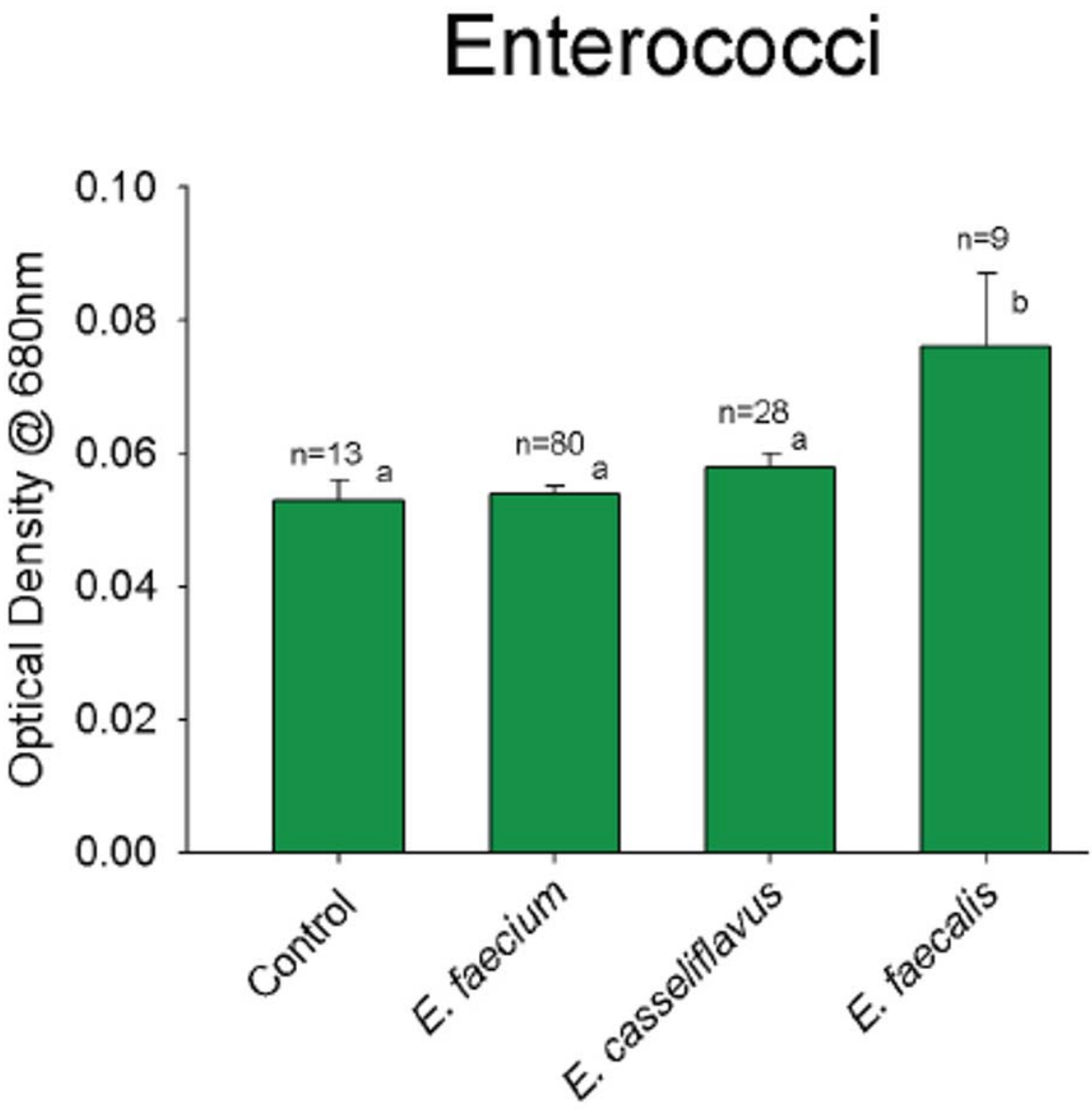

Figure 1. Biofilm formation measured as optical density˜(± SE) after 24 hr incubation for *Enterococcus faecium, Enterococcus casseliflavus*, and *Enterococcus faecalis*. [a, b] Means with different letters differ ($P < .05$)

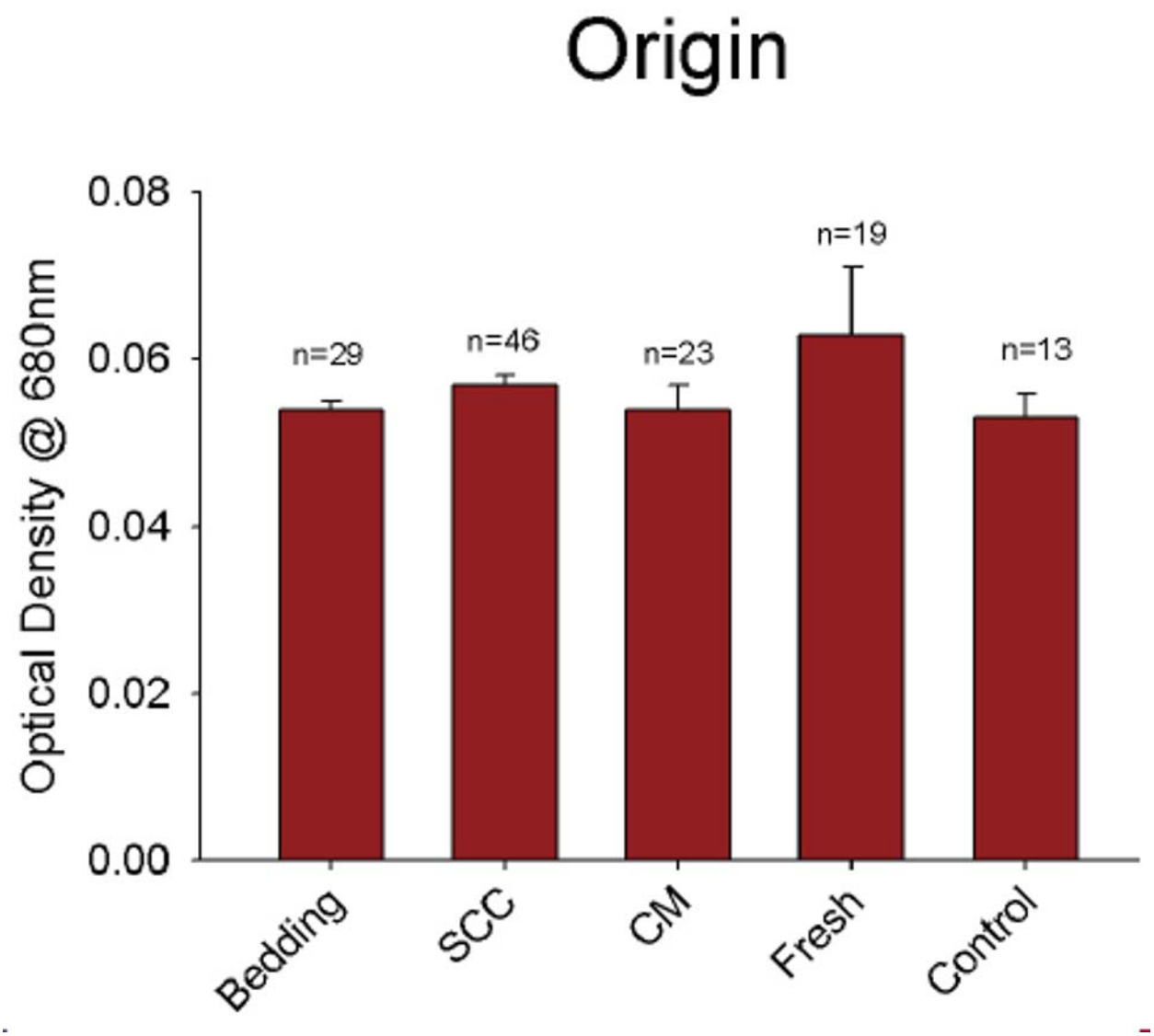

Figure 2. Biofilm formation measured as optical density (± SE) after 24 hr incubation for *Enterococcus* species originating from milk of bovine mammary glands and dried manure bedding.

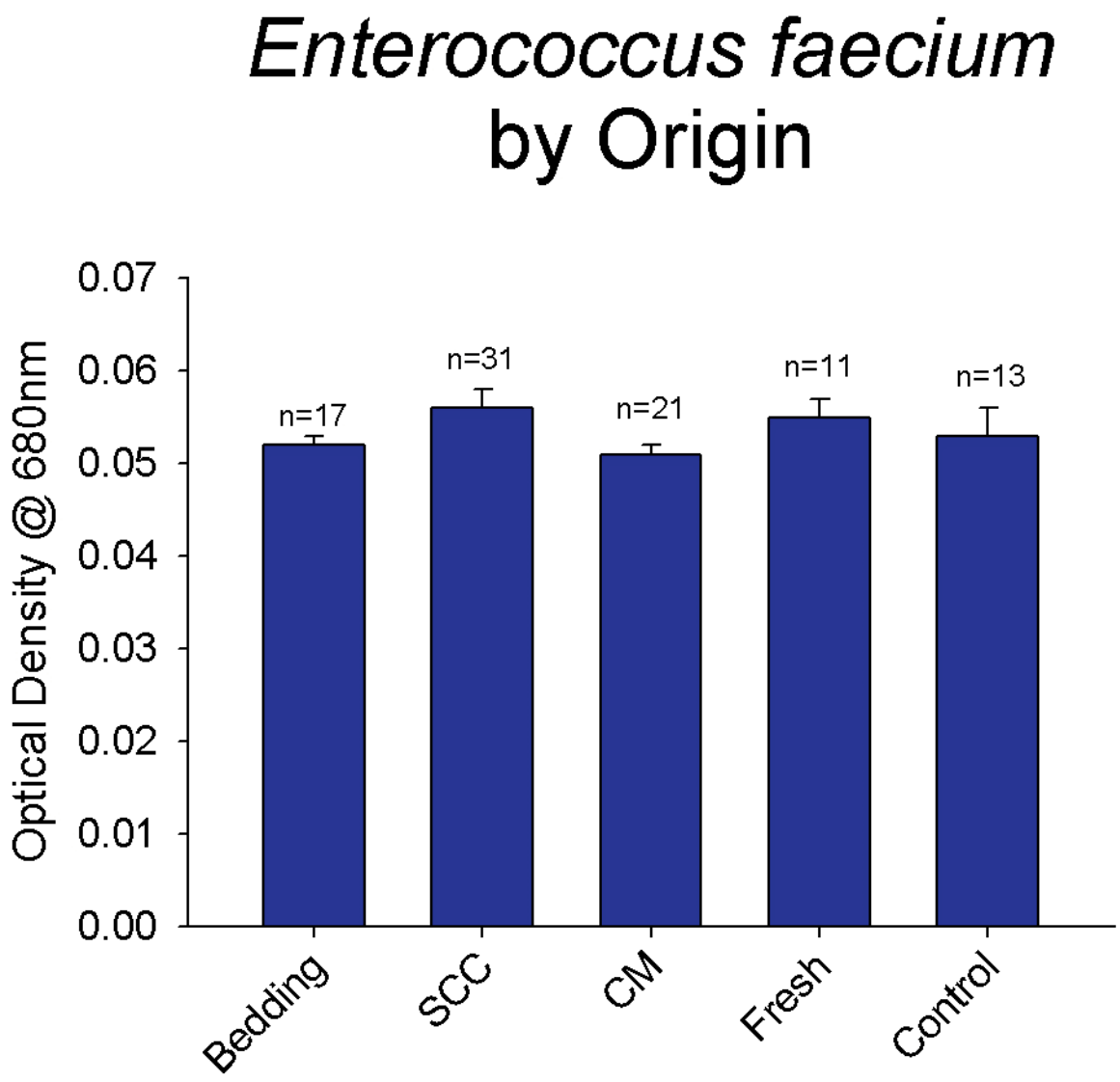

Figure 3. Biofilm formation measured as optical density˜(± SE) after 24 hr incubation for *Enterococcus faecium* by origin of isolates.

CONCLUSIONS

The results of the current survey of *Enterococcus* species of bovine origin

1) lend further support that these bacteria have phenotypic characteristics of environmental opportunist to the mammary gland
2) demonstrate *Enterococcus faecalis* isolates produced biofilm more often than other *Enterococcus* species

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